Two-Photon lightsheet: how to preserve the excitation volume in scattering samples in a selective plane illumination microscope.

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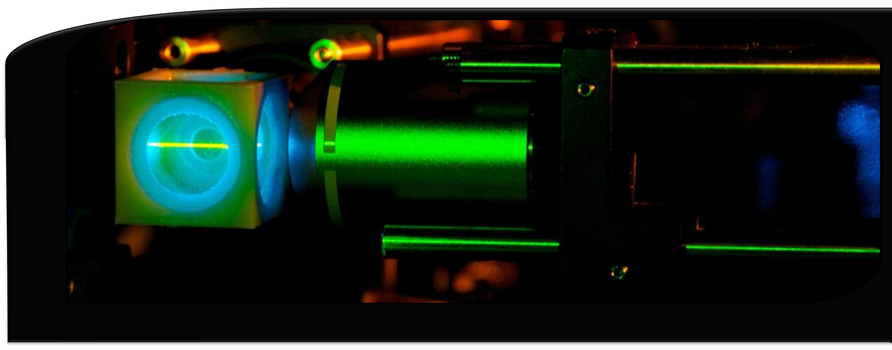


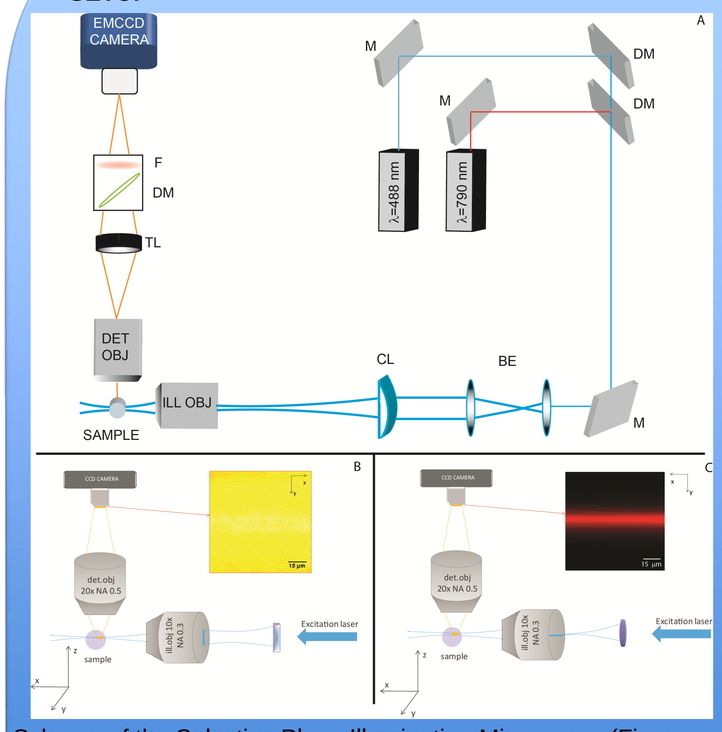
ABSTRACT

In the last years light sheet microscopy has been demonstrated to be an optimal tool for high resolution imaging of large samples such as embryos and tissues. Still, scattering in thick samples could introduce aberrations and distortions of the excitation volume that can decrease the imaging quality. Recently, two-photon excitation microscopy has been coupled with light sheet fluorescence microscopy in order to increase the deep imaging capability of the light-sheet based imaging system(1). Even if two-photon excitation allows the enhancement of the penetration depth capabilities thanks to the use of a higher wavelengths, imaging may still be affected by scattering effects. In fact, scattering produces out-of-focus fluorescence generation, resulting in a shift of the real intensity excitation distribution (2). In this framework, the characterization of scattering based distortions of the excitation volume represent a useful investigation. To this end, we performed measurements of the real light sheet excitation distribution on calibrated phantom samples with tunable optical properties. A comparison between single photon and two photon imaging has been pointed out and results show how effectively two-photon excitation is able to preserve the shape of the excitation light sheet, compared to the single photon case, thus preserving the optical sectioning and the contrast capabilities of the system(3). 3D reconstruction of mammary cell spheroid is performed using two-photon SPIM in order to show the improved imaging capability.

METHODS

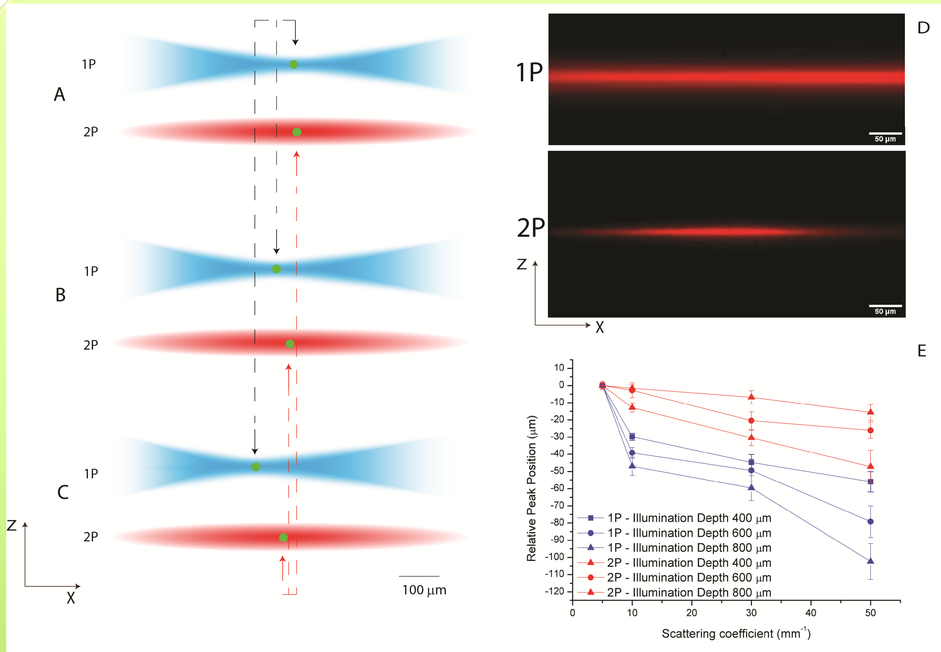
Calibrated Samples: Solution of FITC dextrans (500kDa) & agar gel with various concentration of non fluorescent beads(d=1,03 mm)
Scattering coefficient of 5, 10 ,30 , 50 mm^{-1} concentrations of 3.64, 7.28, 22.75 and 36.4 particles/ml were used.

SETUP



Scheme of the Selective Plane Illumination Microscope (Figure B and fig. C show the adaptation of the system for the experimental measurements. Rotating the cylindrical lens by 90 degrees, the axial section of the intensity excitation distribution appears on the CCD sensor.

CHARACTERISATION OF THE LIGHT-SHEETS



Experiments performed on various phantom samples mimicking different optical properties (5, 10, 30, 50 mm^{-1} respectively). Schematic representation (A-C) of the peak shift (green dot) of the excitation intensity distribution for single photon excitation (blue) and two-photon (red) excitation configurations. Example of the axial section of the measured intensity excitation distributions for the 1P and 2P excitation lightsheets (D) for phantom sample with 5 mm^{-1} scattering coefficient at 400 µm illumination depth. 2PE allows the shift of the uniform region to be reduced (E).

Table 1. Summary of the values obtained from the relative shift of the excitation intensity distributions peaks for both excitation schemes. Scattering coefficient is 50 mm^{-1}.

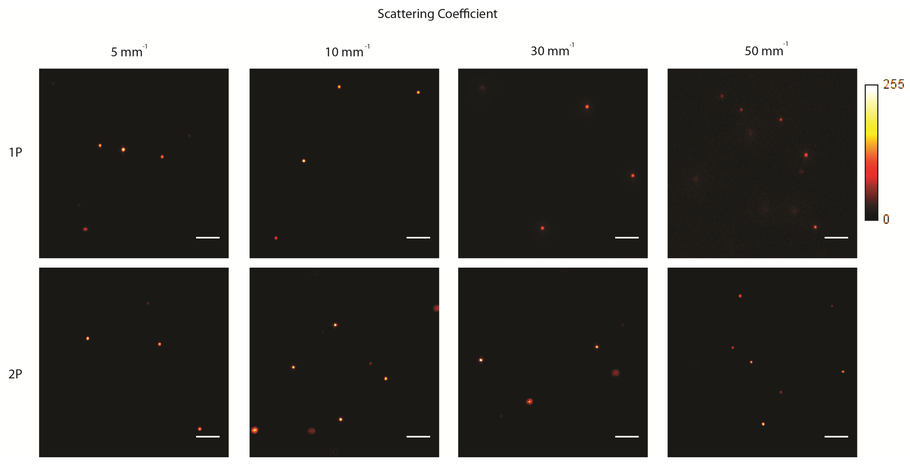
Illumination depth	Distance from ideal Peak Position 1PE	Distance from ideal Peak Position 2PE
400 µm	56 ± 5.9 µm	15.6 ± 4.6 µm
600 µm	79.2 ± 9.2 µm	26 ± 4.8 µm
800 µm	102.4 ± 10.4 µm	47.2 ± 9.5 µm

Excitation Wavelengths: l= 488 nm (1P) and l=790 nm (2P) Intensity used in 1P experiments: I=0.13 kW/cm^2; Intensity used in 2P experiments: I= 19.78 kW/cm^2. Detection Objective: Leica 20x, NA 0.5.

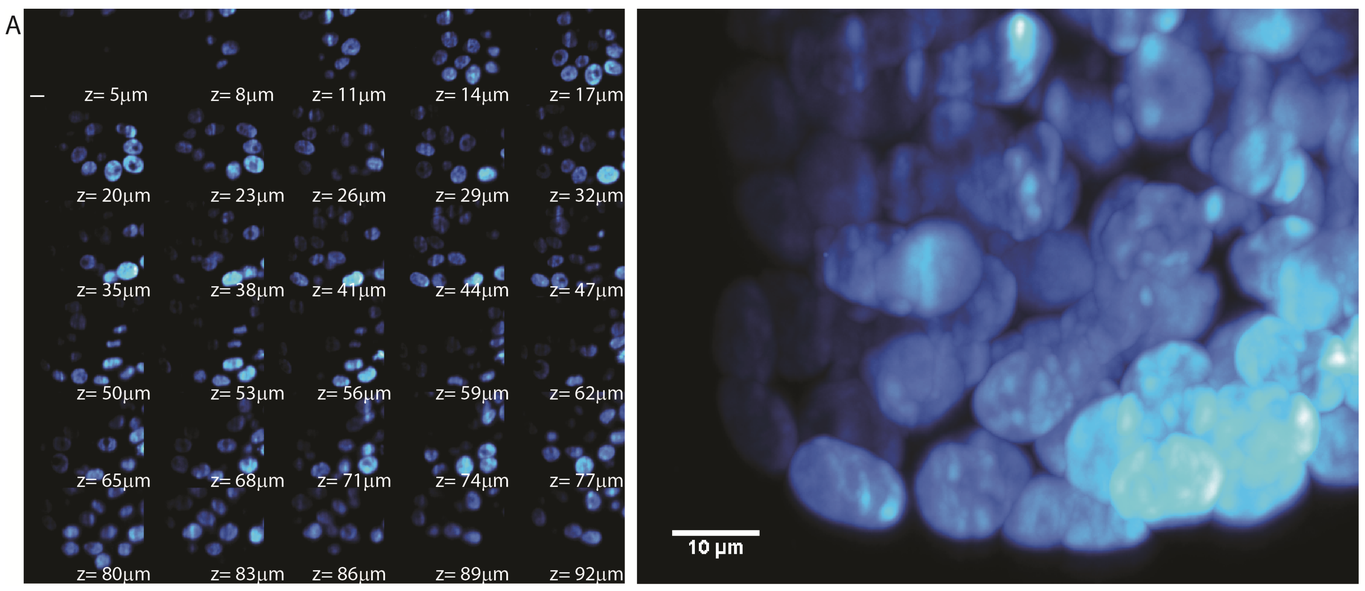
Table 2. Summary of the dimensions of the uniform intensity region within the exciting light sheet. The data shown are the mean values of 15 measurements made on calibrated samples. In the single photon excitation configuration, the area of uniformity reduces significantly by approximately 40 microns, while in the two photon excitation configuration there is no significant variation. This means that the two photon excitation light sheet is preserved more while travelling in deep scattering samples. Data refer to an illumination depth of 400µm.

	Transparent sample (5 mm^{-1})	Scattering sample (50 mm^{-1})
1P uniform light sheet region	428.93 ± 16.34 µm	389.3 ± 20.24 µm
2P uniform light sheet region	292.81 ± 13.66 µm	290.93 ± 19.34 µm

TESTING IMAGING PERFORMANCE



Images of different homogeneously scattering phantom samples with fluorescent beads (diameter=0.17 µm) by means of single photon and two-photon excitation SPIM. All images are acquired at 600 µm illumination depth within the samples. Scale bar is 5 µm . Detection objective Leica HCX APO L U-V-I 12 40X, NA 0.8. Additional magnification introduced:2,5X to get a total magnification of 100X.
Intensity used in single photon experiments: I= 0.11 kW/cm^2; intensity used in 2P experiments: I=19.23 kW/cm^2



Z-stack of mammary epithelial acini (zstep 1 µm) has been acquired . Representative planes within the cell spheroids (spaced 3µm) are shown (A). Scale bar: 10 µm . Maximum intensity projection of the entire volume (B). Excitation wavelength λ=750nm, I= 54.94 KW/cm^2. Objective lens: HCX APO L U-V-I 40x/0.8 WATER.

CONCLUSION

This work sets out to improve knowledge and comprehension of scattering effects both in 1PE and 2PE SPIM. Experiments were performed on calibrated samples, mimicking the various optical properties of some biological samples of interest, such as bladder, muscle, brain and cell aggregates. As expected, the results show how two-photon excitation improves the performances of a lightsheet microscope while imaging scattering samples in depth. Characterisation was performed in terms of maintenance of the uniformity of the excitation intensity distribution and the signal-to-noise ratio. Experiments were carried out at different illumination penetration depths in order to check whether the excitation lightsheet distorts due to light-sample interaction and if significant fluorescence can be generated far from the theoretical focus of the uniform intensity region and 2PE SPIM 3D imaging of mammary cell spheroids was reported. Considering the high scattering coefficient, we found that the two-photon excitation lightsheet gives improved signal to noise ratio while imaging deeper and deeper in a thick sample, thus attracting attention once again to the unique features that a single-plane illumination microscope, coupled with a pulsed laser performing two-photon excitation, demonstrates while imaging thick scattering samples.

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